

*Exhibit B*



0645 '03 FEB 25 P154

BIOGENIC S.A.

AMES II ASSAY (Mix + TA98)

*Ames II Salmonella Mixed Strains + TA98*

Test Articles: APOG/ADS 11/HPS3 & ASCH

Study Number: BIO-1097  
October, 1997

*this is not an  
extract of Padina  
Pavonica +  
is not pertinent  
to this submission.*

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CLIENT RESEARCH LABORATORY REPORT  
Biogenic S.A.  
AMES II ASSAY (Mix + TA98)  
Test Articles: APOG/ADS 11/HPS3 & ASCIII

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## 1. SUMMARY

Test articles APOG/ADS 11/HPS3 and ASCIII were tested in the Ames II *Salmonella* reverse mutation assay (Ames II Assay), using the Ames II Mix and strain TA98 (Ames II Assay, Mix & TA98) in the absence of S9. The constituent strains in the Ames II Mix and strain TA98 are all histidine auxotrophs, and mutagenesis at specific bases in each strain will lead to reversion of the strain (and therefore of the Mix) to histidine prototrophy.

The Ames II Mix & TA98 Assay performed included *Salmonella* strains TA7001, TA7002, TA7003, TA7004, TA7005, and TA7006 in an equimolar mix, and strain TA98. Details (genotype) of each strain are given in section 3.2.

Assays for each *Salmonella* strain were performed in triplicate. Doses of test article APOG/ADS 11/HPS3 in the Ames II Assay (Mix & TA98) were: 0, 57, 114, 229, 458, 916, 1833, and 3667 µg/ml, with concurrent solvent (ethanol) negative controls which gave revertant numbers and frequencies within expected ranges for each strain. Doses of test article ASCIII were: 0, 0.06, 0.13, 0.25, 0.50, 1.0, 2.0, and 4.0 %, with concurrent solvent (H<sub>2</sub>O) negative controls which gave revertant numbers and frequencies within expected ranges for each strain (for expected ranges see Table 1).

*Under the conditions listed above, and using criteria employed to evaluate Ames II Assay (Mix & TA98) data, test articles APOG/ADS 11/HPS3 and ASCIII demonstrated no mutagenic activity when tested with the Ames II Mix or strain TA98, in the absence of S9.*

## 2. INTRODUCTION

The purpose of this study was to assess the mutagenic potential of test articles APOG/ADS 11/HPS3 and ASCIII in the Ames II Assay (Mix + TA98). The six TA7000 series in an equimolar mix and TA98 *Salmonella* strains were employed in the assay, permitting the measurement of 6 different base-substitutions (transitions and transversions), and a frameshift mutation. The assay was performed in the absence of exogenous biotransformation enzymes. The assay is a microplate liquid culture modification of that reported in Gee *et al.* (1994).

### 3. MATERIALS & METHODS

#### 3.1. Test Articles

Test article APOG/ADS 11/HPS3 arrived in three amber vials with black screw caps. Moisture was apparent on the walls of the vials. The vials had a white printed label:

Lot: 5456W1  
APOG/ADS 11/HPS3

The test article was stored dessicated at 4°C.

Test article ASCIII arrived in four 2.5 ml clear glass ampules. They had a white printed label:

Lot: 3385  
ASCIII

The test article was stored at room temperature, in the dark.

#### 3.2. Bacterial Strains.

Table 1 shows the Ames II Assay (Complete) strains, a subset of which were employed in this assay.

Table 1. AMES II Strains

Strain <sup>a</sup>	Target <sup>b</sup>	Mutation <sup>c</sup>	rfa <sup>d</sup>	uvrB <sup>e</sup>	pKM101 <sup>f</sup>	rev/10 <sup>8</sup> <sup>g</sup>	QC rev/48 wells <sup>h</sup>
TA7001	bp subst. <sup>i</sup>	A:T→G:C	✓	✓	✓	0 - 5	0 - 3
TA7002	bp subst.	T:A→A:T	✓	✓	✓	0 - 9	0 - 5
TA7003	bp subst.	T:A→G:C	✓	✓	✓	0 - 1	0 - 1
TA7004	bp subst.	G:C→A:T	✓	✓	✓	0 - 20	0 - 10
TA7005	bp subst.	G:C→T:A	✓	✓	✓	0 - 20	0 - 10
TA7006	bp subst.	C:G→G:C	✓	✓	✓	0 - 5	0 - 5
Mix	bp subst.	All above	✓	✓	✓	0 - 20	0 - 10
TA98	frameshift	+G:C	✓	✓	✓	0 - 35	0 - 17
TA1537	frameshift	+G:C	✓	✓	×	0 - 35	0 - 17

<sup>a</sup> Base change detected by this strain

<sup>b</sup> Defective lipopolysaccharide cell wall, permitting greater access for larger molecules

<sup>c</sup> Defective in DNA repair

<sup>d</sup> Plasmid which enhances error-prone repair & confers Ampicillin resistance

<sup>e</sup> Spontaneous revertants per 10<sup>8</sup> viable cells

<sup>f</sup> Spontaneous revertants required for Quality Control acceptance. Slightly higher spontaneous levels are, in many cases still passed on variance.

<sup>g</sup> base pair substitution detected

✓ present, × absent

### 3.3. Metabolic Activation

The assay performed in this report (Ames II, Mix + TA98), was done so in the absence of S9 fraction and therefore did not include exogenous metabolic activation.

### 3.4. Positive Controls

The following positive control chemicals were used in assessing the performance of the reported Ames II Assays.

Table 2. Positive Controls

Ames II Strain	Control Chemical	Conc.
Mix	4-Nitroquinoline N-oxide (4-NQO)	0.06-0.5 µg/ml
TA98	2-nitrofluorene (2NF)	0.25-2.0 µg/ml

### 3.5. Test Article Preparation

A 25X stock solution of the test articles was prepared as listed below:

Table 3.

Test Article	Solvent	Stock Concentration
APOG/ADS 11/HPS3	ethanol	91.5 mg/ml
ASCIH	dd H <sub>2</sub> O	100%

### 3.6. Ames II Assay

The assay protocol is described in Appendix 1.

Test article APOG/ADS 11/HPS3 was tested against the Mix and TA98 *Salmonella* strains at 8 dose levels (including a zero dose). Each dose was performed in triplicate using independent cultures.

The doses employed were: 0, 57, 114, 229, 458, 916, 1833, and 3667 µg/ml.

Test article ASCIH was tested against the Mix and TA98 *Salmonella* strains at 8 dose levels (including a zero dose). Each dose was performed in triplicate using independent cultures.

The doses employed were: 0, 0.06, 0.13, 0.25, 0.50, 1.0, 2.0, and 4.0 %.

### 3.6.1. Dosing Protocol

In one well of a 24 well plate (one well/strain/dose/replicate) 0.190 ml of Exposure Medium was aliquoted. To this was added 0.050 ml of culture, giving a total volume of 0.240 ml. To each of these cultures, 0.010 ml of test article, diluted to the appropriate concentration was added, to give a total of 0.250 ml. This mixture was then incubated for 90 minutes at 37°C with agitation at 250 rpm.

At the conclusion of the 90 minute incubation, each well received 2.8 ml of *Salmonella* Indicator Medium and was mixed briefly before being distributed in 0.05 ml aliquots to 48 wells of a 384-well microtiter plate. One plate was used/strain/replicate.

Plates were then incubated at 37°C for 48 hours, after which yellow (positive) wells were counted.

### 3.6.2. Determination of Colony Number

From triplicate data sets, the mean number of positive wells for each strain at each dose was calculated.

### 3.6.3. Determination of Fold Increase

The fold increase of bacterial revertant colonies compared to the background (zero dose) revertant colony number was determined by dividing the mean number of colonies at each dose by that at the zero dose. Student's *t*-tests were used to determine significance (at the  $\alpha = 0.05$  level) for fold inductions greater than 3.0. The Student's *t*-test was performed to test the null hypothesis for every concentration of compound in comparison to the zero-dose control. Because the samples have different variance, the following formula was used:

$$t = \frac{[\bar{x}_1 - \bar{x}_2]}{\sqrt{\left[\frac{S_1^2}{n_1}\right] + \left[\frac{S_2^2}{n_2}\right]}}$$

where:

$\bar{x}_1$  = the mean colony count for sample 1 (zero dose control)

$\bar{x}_2$  = the mean colony count for sample 2 (concentration X)

$S_1^2$  = the variance of colony count 1

$S_2^2$  = the variance of colony count 2

$n_1$  = the number of replicate colony counts for count 1 ( $n = 3$ )

$n_2$  = the number of replicate colony counts for count 2 ( $n = 3$ )

Since the *t*-test was used to evaluate means *greater* than the control value, reference was made to a table of critical values for the Student's *t*-test which records an area of  $\alpha$  in the right hand tail only.



The degrees of freedom value was determined by the smaller of the values  $n_1-1$  or  $n_2-1$ , both of which equal 2.

Although statistical analyses have been applied to all data collected, fold increases in revertant numbers in the Ames II Assay are not classified as positive if less than 3.0. Below this fold increase value, the data are unreliable with respect to determining mutagenicity. To be classified as a mutagen, a compound is therefore required to yield a reproducible fold increase of greater than 3.0, or to show a clear dose response.

#### 3.6.4. *Data Analysis*

The mean number of positive wells and fold increase were determined as described above. A positive result in the Ames II Assay requires a fold increase value of greater than 3.0.

#### 3.6.5. *Archived Data*

Raw data for this study are located in Xenometrix laboratory notebooks as follows:

Table 4.

Notebook	User	Pages
162/TRL	Sally Haugen	1-2

#### 4. RESULTS

Table 5. Test Article APOG/ADS11/HPS3

Mix

Conc. (ug/ml)	n	mean # pos. wells	SD	Fold Increase	t-test (2DF) alpha = 0.05
0	3	1.67	0.58	-	-
57	3	0.67	1.15	0.40	1.34
115	3	1.67	2.08	1.00	0.00
229	3	0.33	0.58	0.20	2.83
458	3	0.33	0.58	0.20	2.83
916	3	0.00	0.00	0.00	5.00
1,833	3	0.00	0.00	0.00	5.00
3667	3	0.00	0.00	0.00	5.00

<sup>a</sup> number of replicates

<sup>b</sup> mean number of yellow wells counted over three replicate 48-well portions of a 384 well plate.

<sup>c</sup> standard deviation

<sup>d</sup> mean number of positive wells at each dose, divided by the mean number of positive wells at the zero dose control.

<sup>e</sup> Student's t-test significant at the 0.05 level if calculated value is greater than 2.92 (2 degrees of freedom).

Test article APOG/ADS11/HPS3 showed no evidence of mutagenicity when assayed with the Ames II Mix, in the absence of S9 fraction. Raw plate counts for Table 5 are shown in Appendix 2, Table 11.

Table 6. Test Article APOG/ADS11/HPS3

TA98

Conc. (ug/ml)	n	mean # pos. wells	SD	Fold Increase	t-test (2DF) alpha = 0.05
0	3	1.67	1.53	-	-
57	3	3.00	2.65	1.80	0.76
115	3	1.33	1.15	0.80	0.30
229	3	1.67	1.15	1.00	0.00
458	3	1.00	0.00	0.60	0.76
916	3	1.00	1.00	0.60	0.63
1,833	3	1.00	1.00	0.60	0.63
3667	3	0.00	0.00	0.00	1.89

Test article APOG/ADS11/HPS3 showed no evidence of mutagenicity when assayed with TA98, in the absence of S9 fraction. Raw plate counts for Table 6 are shown in Appendix 2, Table 12.

Table 7. Test Article ASCIII

Mix

Conc. (%)	n	mean # pos. wells	SD	Fold Increase	t-test (2DF) alpha = 0.05
0.00	3	0.00	0.00	-	-
0.06	3	1.00	0.00	-	-
0.13	3	0.33	0.58	-	1.00
0.25	3	0.67	0.58	-	2.00
0.50	3	1.00	1.00	-	1.73
1.00	3	0.67	1.15	-	1.00
2.00	3	0.67	1.15	-	1.00
4.00	3	2.33	0.58	-	7.00

Test article ASCIII showed no evidence of mutagenicity when assayed with the Ames II Mix, in the absence of S9 fraction. Although the zero spontaneous value observed for the Mix does not allow for the calculation of fold induction values, the marginal increase in absolute numbers, in combination with the absence of a clear dose response, suggests that test article ASCIII is negative in the Ames II Mixed Strains. Raw plate counts for Table 7 are shown in Appendix 2, Table 13.

Table 8. Test Article ASCIII

TA98

Conc. (%)	n	mean # pos. wells	SD	Fold Increase	t-test (2DF) alpha = 0.05
0.00	3	2.00	2.65	-	-
0.06	3	1.00	1.00	0.50	0.61
0.13	3	1.00	0.00	0.50	0.65
0.25	3	2.33	1.53	1.17	0.19
0.50	3	2.00	1.00	1.00	0.00
1.00	3	2.33	1.53	1.17	0.19
2.00	3	1.67	1.53	0.83	0.19
4.00	3	1.67	1.53	0.83	0.19

Test article ASCIII showed no evidence of mutagenicity when assayed with TA98, in the absence of S9 fraction. Raw plate counts for Table 8 are shown in Appendix 2, Table 14.

Table 9. Positive Controls (plate counts)

Mix		TA98	
2NF/4NQO (ug/ml)	# revertants	2NF/4NQO (ug/ml)	# revertants
0.25/0.0625	24	0.25/0.0625	18
0.5/0.125	34	0.5/0.125	26
1/0.25	47	1/0.25	39
2/5	47	2/5	47

## 5. DISCUSSION

All negative controls (zero dose solvent controls) run concurrently with the assays gave revertant colony numbers within expected limits.

Quality Control appropriate positive controls gave revertant colony numbers within expected limits.

No statistically significant increases in revertant yield were seen in the Ames II Mix or Strain TA98 in the absence of S9.

## 6. CONCLUSION

Using the experimental conditions outlined in this report, test articles APOG/ADS 11/HPS3 and ASCIII are not mutagenic in the Ames II Mix or strain TA98 in the absence of S9.

## 7. REFERENCES

- Gee, P., Maron, D., and Ames, B.N., (1994) Detection and classification of mutagens: A set of base-specific *Salmonella* tester strains *Proc. Natl. Acad. Sci. (USA)* 91, 11606-610.
- Vennit, S., Crofton-Sleigh, C., and Foster, R., (1984) In: *Mutagenicity Testing*, pp45 (S. Vennit and J.M. Parry. eds), IRL Press. Oxford, UK.

## Appendix 1: Assay Outline

### AMES II ASSAY: Version 2.0

#### *General Description*

The Ames II Assay consists of six new *Salmonella typhimurium* tester strains, each of which detects only one of the six possible base pair substitution mutation types, allowing the generation of mutational spectra data without the need for DNA sequence analysis. Because these strains revert only by specific mutational events, spontaneous reversion frequencies are approximately 10 to 100-fold lower than those typically observed with traditional *Salmonella* tester strains such as TA100 or TA102, which have a range of possible reversion pathways. Ames II strain reversion frequencies are shown in Table 9. The minimal genetic complementation between the six TA7000 series strains means that they may be combined in a single assay in order to screen for moderate to strong mutagens if there is no requirement for mutational spectra data. The tester strains TA98 and TA1537 are included in the full Ames II Assay (Ames II complete) for the detection of frameshift-inducing mutagens. However, in the rapid Ames II screen (Ames II (Mix + TA98)), only the more sensitive TA98 is used.

The new *Salmonella* tester strains TA7001-TA7006 have been modified for maximum sensitivity to test compound. These modifications include:

- Deletion of the excision repair gene *uvrB*. This mutation prevents the removal of bulky adducts from DNA.
- Mutation of a gene required for synthesis of the bacterial cell wall (*rfa*) which increases permeability of *Salmonella* to test compound.
- Introduction of the episome pKM101, which carries the *umuDC* homologues *mucA/B*. These gene products increase the cells ability to perform mutagenic lesion bypass repair during DNA replication.

Table 10. Ames II Strains

Strain	Target	Mutation <sup>a</sup>	rfa	uvrB	pKM101	rev/10 <sup>8</sup> <sup>b</sup>
TA7001	bp subst. <sup>c</sup>	A:T→G:C	✓	✓	✓	0 - 5
TA7002	bp subst.	T:A→A:T	✓	✓	✓	0 - 9
TA7003	bp subst.	T:A→G:C	✓	✓	✓	0 - 1
TA7004	bp subst.	G:C→A:T	✓	✓	✓	0 - 20
TA7005	bp subst.	G:C→T:A	✓	✓	✓	0 - 20
TA7006	bp subst.	C:G→G:C	✓	✓	✓	0 - 5
Mix	bp.subst.	All above	✓	✓	✓	0 - 20
TA98	frameshift	-GC	✓	✓	✓	0 - 35
TA1537	frameshift	-GC	✓	✓	x	0 - 35

<sup>a</sup> Base change detected by this strain

<sup>b</sup> Revertants per 10<sup>8</sup> viable cells

<sup>c</sup> Base-pair substitutions detected

✓ The given characteristic is present in this strain

x The given characteristic is absent from this strain

### *Assay Procedure*

Because of the low spontaneous reversion frequencies for the TA7000 strains and their associated mixture, the assay has been converted to a modified fluctuation test using 384 well (48 wells per sample, per dose) microtiter plates. The frameshift tester strains are not combined with the Ames II Mix, but are assayed concurrently in microtiter plates.

The modified Ames II fluctuation test, in brief, is as follows:

1. Pre-growth of tester strains overnight in oxoid broth.
2. A 90 minute incubation ( $10^8$  cells x 1 generation) in Exposure Medium with limiting histidine (1.0 mg/ml) in the presence of toxicant and S9 if employed.
3. Dilution and plating of cells in medium which selects for revertants. This medium contains a pH indicator dye which turns color from purple to yellow upon colony growth.
4. Incubation of the microtiter plates for 48 hours to allow growth of revertant colonies.
5. Scoring of microtiter plates for positive (yellow) wells, data entry, and evaluation of mutagenic potential.

## Appendix 2: Raw Data

### *Plate counts*

Table 11. Test Article APOG/ADS11/HPS3

Mix			
Conc. (ug/ml)	Replicate #1	Replicate #2	Replicate #3
0	2	2	1
57	2	0	0
115	0	1	4
229	1	0	0
458	0	1	0
916	0	0	0
1,833	0	0	0
3,667	0	0	0

Table 12. Test Article APOG/ADS11/HPS3

TA98			
Conc. (ug/ml)	Replicate #1	Replicate #2	Replicate #3
0	2	3	0
57	1	2	6
115	2	2	0
229	1	3	1
458	1	1	1
916	2	0	1
1,833	0	1	2
3,667	0	0	0

Table 13. Test Article ASC III

Mix			
Conc. (%)	Replicate #1	Replicate #2	Replicate #3
0.00	0	0	0
0.06	1	1	1
0.13	0	1	0
0.25	0	1	1
0.50	2	1	0
1.00	0	0	2
2.00	0	2	0
4.00	2	3	2

Table 14. Test Article ASC III

TA98			
Conc. (%)	Replicate #1	Replicate #2	Replicate #3
0.00	5	1	0
0.06	0	1	2
0.13	1	1	1
0.25	2	1	4
0.50	2	3	1
1.00	1	4	2
2.00	0	2	3
4.00	0	3	2